Programmable DNA cleavage by Ago nucleases from mesophilic bacteria *Clostridium butyricum* and *Limnothrix rosea*

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Supplementary Information

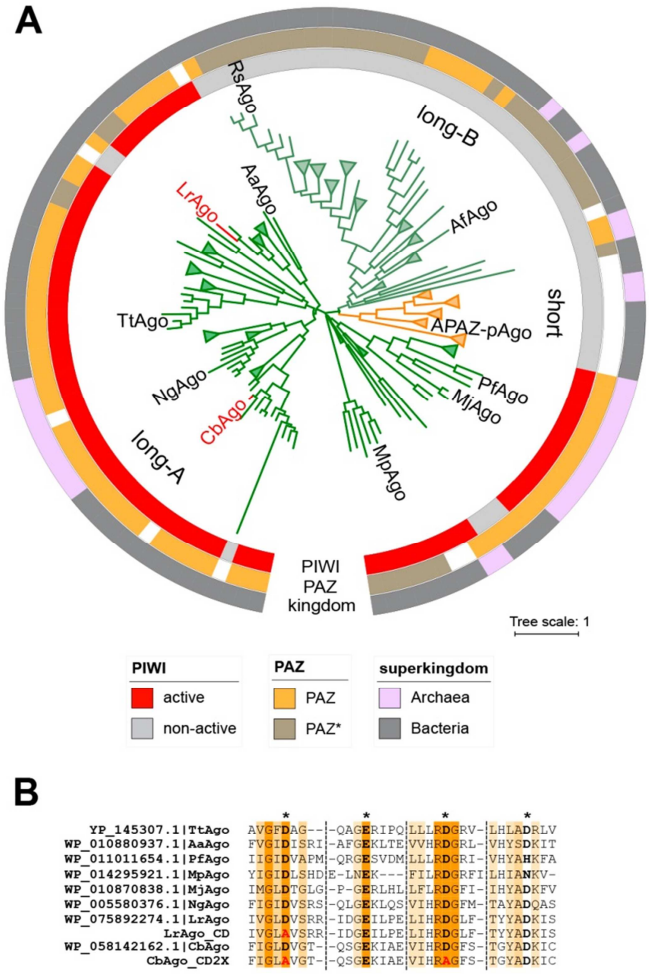

Figure S1. CbAgo and LrAgo are mesophilic pAgo proteins with an intact catalytic tetrad.
(A) The circular phylogenetic tree of nonredundant set of pAgos constructed based on multiple alignment of the MID-PIWI domains (4). The pAgo proteins were annotated as follows (from the inner to the outer circles): the type of the PIWI domain, depending on the presence of the catalytic tetrad DEDX; the presence and the type of the PAZ domain (full-length PAZ, ochre; PAZ* - incomplete PAZ lacking a part of the nucleic acid binding pocket, brownish; no PAZ, white; APAZ, analog of PAZ domain); the superkingdom to which the corresponding pAgo belongs. Long-A, Long-B, and short-pAgo clades are indicated (4-6). The scale bar represents the evolutionary rate calculated under the JTT+CAT evolutionary model. Positions of biochemically characterized pAgos are shown; CbAgo and LrAgo are shown in red.
(B) Multiple sequence alignment of conserved amino acid residues (marked by color and asterisks) of the DEDX tetrad localized in the PIWI domain of pAgos. The catalytically dead variants of the LrAgo and CbAgo proteins (LrAgo_CD and LrAgo_CD2X) with amino acid substitutions within the catalytic tetrad are also shown. Aa, *Aquifex aeolicus*; Af, *Archaeoglobus fulgidus*; Cb, *Clostridium butirycum; Lr, Limnothrix rosea*; Mj, *Methanocaldococcus jannaschii*; Mp, *Marinitoga piezophila*; Ng, *Natronobacterium gregoryi*; Pf, *Pyrococcus furiosus*; Rs, *Rhodobacter sphaeroides*; Tt, *Thermus thermophilus.*

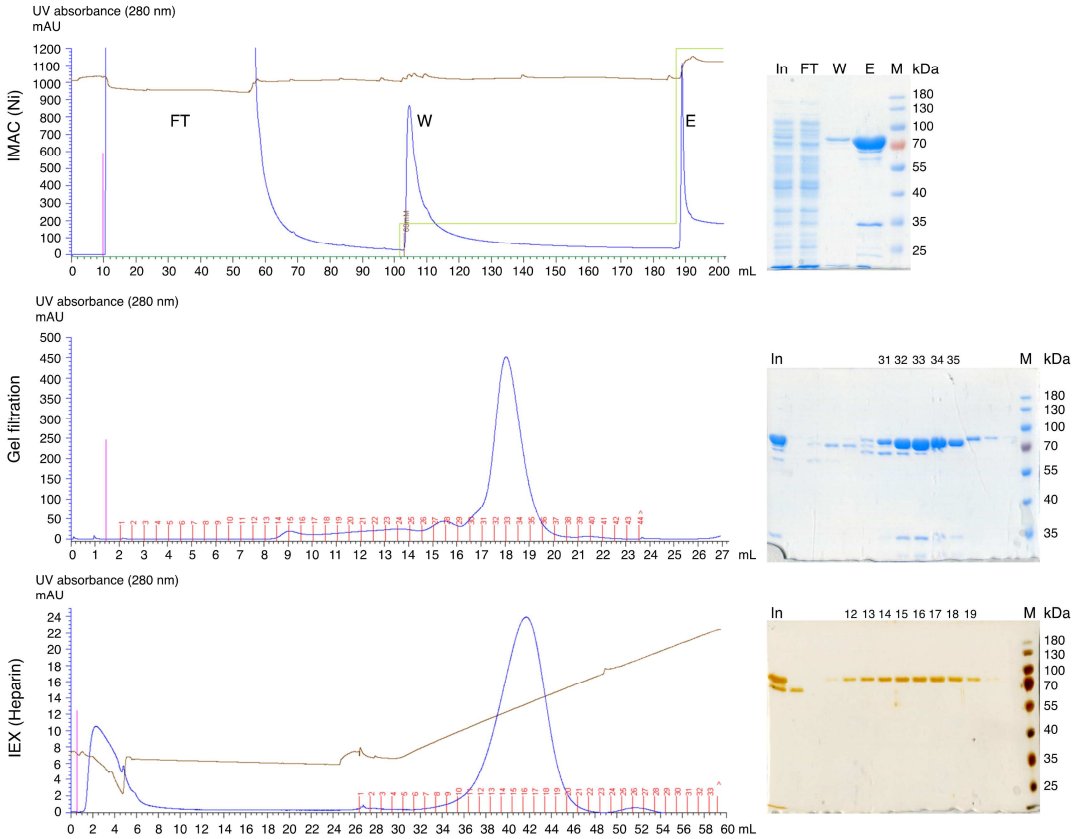

Figure S2. Three-step purification of LrAgo.
(*Upper panel*) Ni-NTA-column chromatogram (*left*, UV absorbance at 280 nm is shown in arbitrary units) and a representative SDS-PAGE gel of indicated fractions (*right*; In, input, FT, flowthrough; W, 60 mM imidazole wash; E, 270 mM imidazole elution; M, marker). (*Middle panel*) Gel filtration chromatogram (*left*) and a representative gel of indicated fractions (In, input; 31-35, chromatography fractions containing LrAgo). (*Lower panel*) Chromatography on a Heparin column (*left*) and a representative gel of indicated fractions (silver staining).

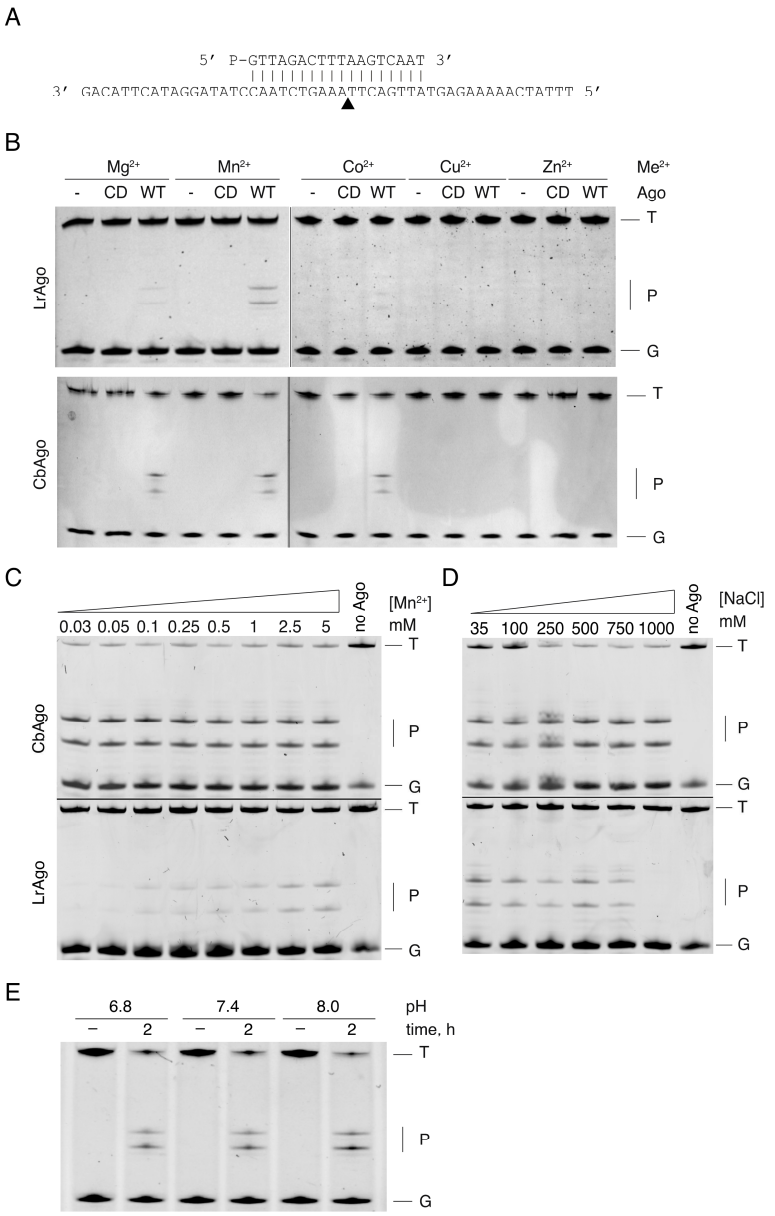

Figure S3. Biochemical properties of LrAgo and CbAgo.
(A) Guide and target DNA oligonucleotides used in the assays. Black triangle indicates the cleavage site. (B) Effects of different cations on LrAgo (top) and CbAgo (bottom) activity. The experiments were performed with wild-type (WT) and catalytically dead (CD) pAgo variants in reaction buffers containing indicated divalent cations at 5 mM concentration. (C) Dependence of the cleavage efficiencies of CbAgo (top) and LrAgo (bottom) on Mn^{2+} concentration. (D) Target DNA cleavage at different NaCl concentrations. (E) pH-dependence of ssDNA cleavage by LrAgo. The reactions were performed in Tris-HCl buffer solutions with different pH values. All reactions were carried out for 2 hours at 37 °C, at the 5:5:1 pAgo:guide:target molar ratio (500 nM pAgo preloaded with 500 nM guide DNA, plus 100 nM target DNA). Positions of the guide (G), target (T) and cleavage products (P) are indicated.

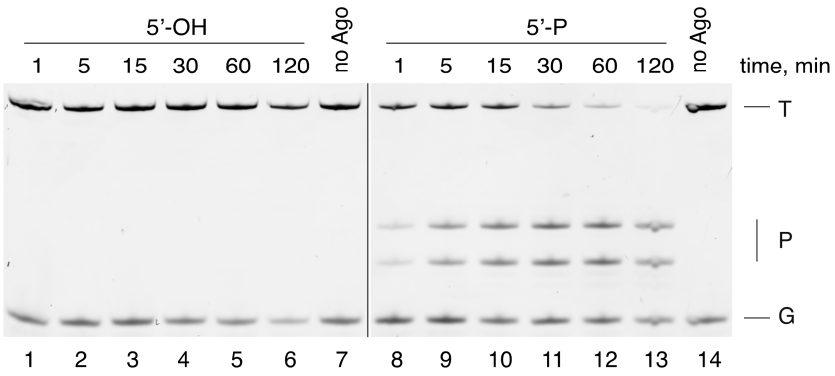

Figure S4. Analysis of ssDNA cleavage by CbAgo loaded with complementary 5'-OH and 5'-P guides at 55°C. (A) The reactions were performed at the 5:2:1 pAgo:guide:target molar ratio (single turnover conditions) for indicated time periods, SYBR Gold staining. The absence of activity in reactions with 5'-OH guides suggests the inability of CbAgo to utilize nonphosphorylated DNA guides at 55 °C.

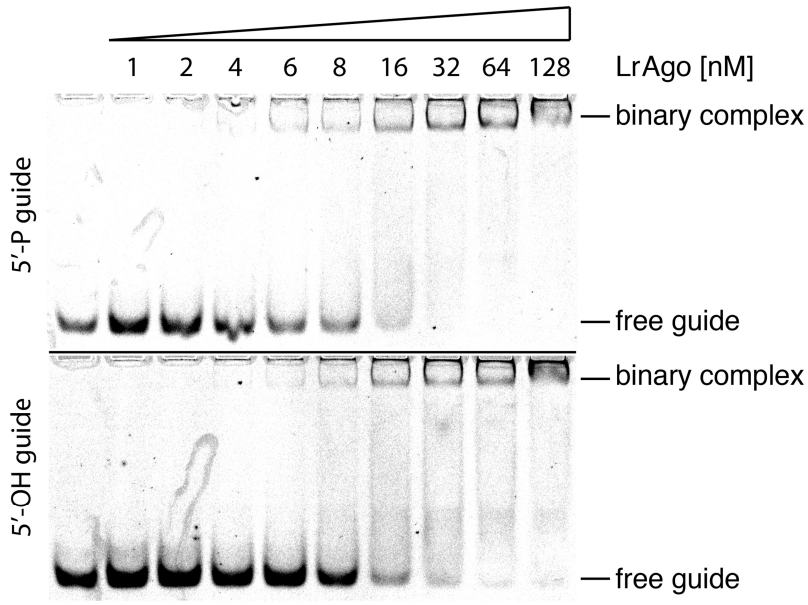

Figure S5. LrAgo forms binary complexes with 5'-P and 5'-OH guides with comparable affinities. Guide DNA (5 nM) was incubated with increasing amounts of LrAgo at 37°C for 10 minutes and resolved by native 10% PAGE. Positions of the free guide and the binary complex are indicated. EMSA was conducted in quadruplicate and a representative gel is shown.

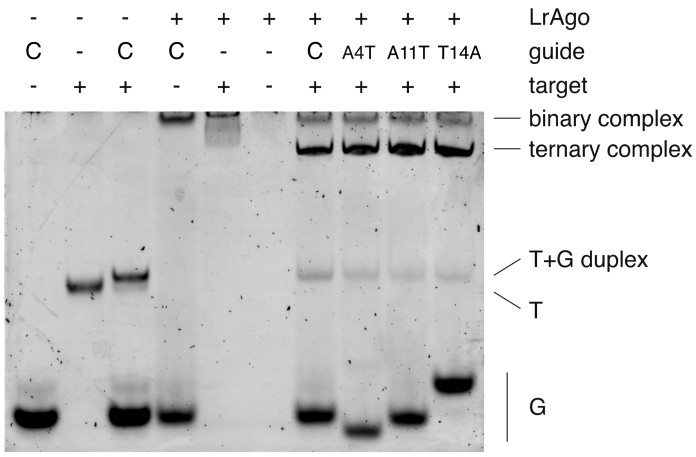

Figure S6. Formation of binary and ternary complexes with guide and target DNAs by LrAgo. The complexes were formed with 500 nM Ago, 500 nM guide DNA and 100 nM target DNA for 10 minutes at 37 °C and resolved by 10% native PAGE. Mismatches in guide DNA that significantly alter the efficiency of LrAgo-mediated cleavage (Fig. 5) do not affect target binding at the given time-scale. C, fully complementary guide DNA; T, target DNA; G, guide DNA; nucleotide substitutions in the mismatched guides are designated above the gel.

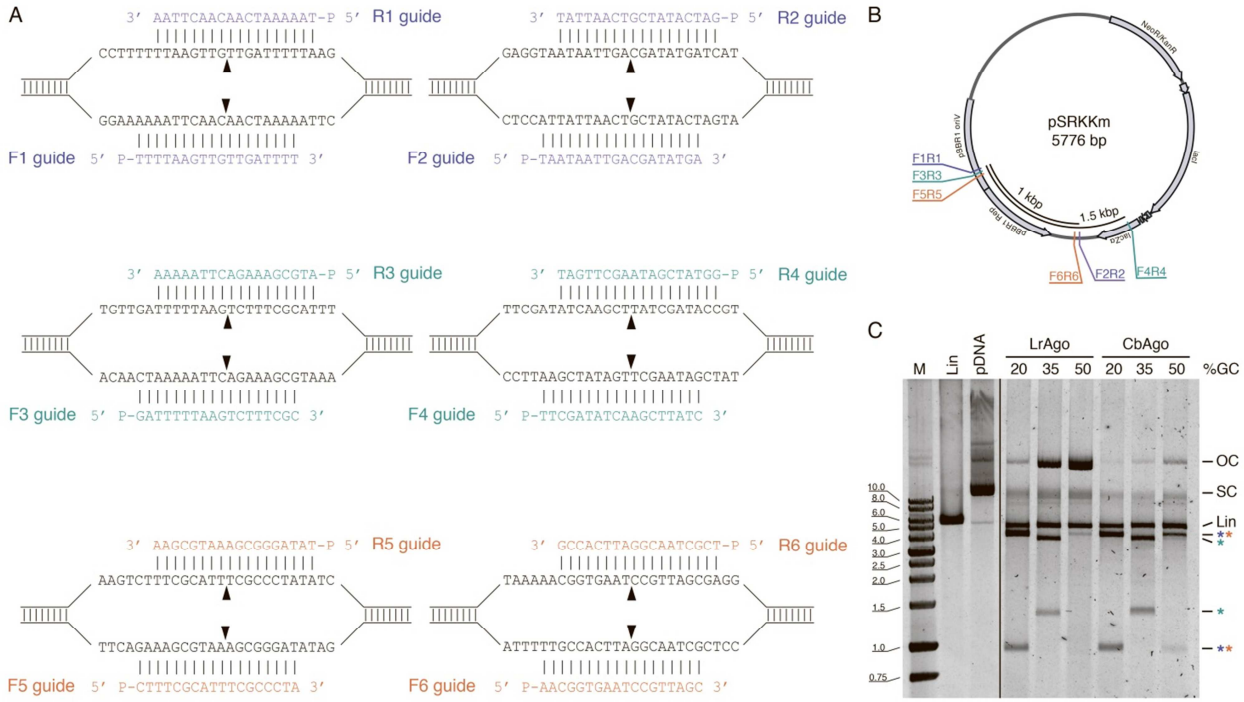

Figure S7. Analysis of plasmid DNA cleavage by CbAgo and LrAgo at DNA sites with different GC content. (A) Six regions in the pSRKKm plasmid with different GC content targeted by different pairs of DNA guides (F1R1, F2R2, F3R3, F4R4, F5R5, F6R6). Black triangles indicate the predicted cleavage sites. (B) Scheme of the pSRKKm plasmid with the positions of corresponding guide pairs shown in colour. The lengths of specific cleavage products are indicated. (C) Plasmid cleavage by LrAgo and CbAgo at 55 °C. The reactions were performed with guide pairs with indicated GC content for 2 hours and resolved by agarose gel-electrophoresis, followed by SYBR Gold staining. 200 nM pAgo, 50 nM each guide DNA and 2 nM plasmid DNA were taken in the reaction. FR, forward and reverse guide DNAs; M, molecular weight marker; Lin, control linear plasmid obtained after treatment with a restriction endonuclease (KpnI); LIN, linearized plasmid; OC, open circular plasmid; SC, supercoiled plasmid; positions of specific cleavage products are indicated with asterisks.

Table S1. Sequences of oligonucleotides used in the cleavage assays.

Oligonucleotide name	Sequence (5'-3')	Description
1. G-guide	GTTAGACTTTAAGTCAAT	guide forms 5'-G pair with G-tDNA
2. C-guide	CTTAGACTTTAAGTCAAT	guide forms 5'-C pair with C-tDNA
3. A-guide	ATTAGACTTTAAGTCAAT	guide forms 5'-A pair with A-tDNA
4. T-guide	TTTAGACTTTAAGTCAAT	guide forms 5'-T pair with T-tDNA
5. g38NT_mm1	CTTAGACTTTAAGTCAAT	guide forms mismatched pair in position 1 with G-tDNA
6. g38NT_mm2	GATAGACTTTAAGTCAAT	guide forms mismatched pair in position 2 with G-tDNA
7. g38NT_mm3	GTAAGACTTTAAGTCAAT	guide forms mismatched pair in position 3 with G-tDNA
8. g38NT_mm4	GTTTGACTTTAAGTCAAT	guide forms mismatched pair in position 4 with G-tDNA
9. g38NT_mm5	GTTACACTTTAAGTCAAT	guide forms mismatched pair in position 5 with G-tDNA
10. g38NT_mm6	GTTAGTCTTTAAGTCAAT	guide forms mismatched pair in position 6 with G-tDNA
11. g38NT_mm7	GTTAGAGTTTAAGTCAAT	guide forms mismatched pair in position 7 with G-tDNA
12. g38NT_mm8	GTTAGACATTAAGTCAAT	guide forms mismatched pair in position 8 with G-tDNA
13. g38NT_mm9	GTTAGACTATAAGTCAAT	guide forms mismatched pair in position 9 with G-tDNA
14. g38NT_mm10	GTTAGACTTAAAGTCAAT	guide forms mismatched pair in position 10 with G-tDNA
15. g38NT_mm11	GTTAGACTTTTAGTCAAT	guide forms mismatched pair in position 11 with G-tDNA
16. g38NT_mm12	GTTAGACTTTATGTCAAT	guide forms mismatched pair in position 12 with G-tDNA
17. g38NT_mm13	GTTAGACTTTAACTCAAT	guide forms mismatched pair in position 13 with G-tDNA
18. g38NT_mm14	GTTAGACTTTAAGACAAT	guide forms mismatched pair in position 14 with G-tDNA
19. g38NT_mm15	GTTAGACTTTAAGTGAAT	guide forms mismatched pair in position 15 with G-tDNA
20. g38NT_mm16	GTTAGACTTTAAGTCTAT	guide forms mismatched pair in position 16 with G-tDNA
21. g38NT_mm17	GTTAGACTTTAAGTCATT	guide forms mismatched pair in position 17 with G-tDNA
22. g38NT_mm18	GTTAGACTTTAAGTCAAA	guide forms mismatched pair in position 18 with G-tDNA
23. G-tDNA	TTTATCAAAAAGAGTATTGACTTAAAG TCTAACCTATAGGATACTTACAG	50 nt target DNA for G-guide
24. C-tDNA	TTTATCAAAAAGAGTATTGACTTAAAG TCTAAGCTATAGGATACTTACAG	50 nt target DNA for C-guide
25. A-tDNA	TTTATCAAAAAGAGTATTGACTTAAAG	50 nt target DNA for A-guide

	TCTAATCTATAGGATACTTACAG	
26. T-tDNA	TTTATCAAAAAGAGTATTGACTTAAAG TCTAAACTATAGGATACTTACAG	50 nt target DNA for T-guide
27. RNA guide	GUUAGACUUUAAGUCAAU	18 nt RNA guide for the guide/target specificity assay
28. RNA target	UUUAUCAAAAAGAGUAUUGACUUAAA GUCUAACCUAUAGGAUACUUACAG	50 nt RNA target for the guide/target specificity assay
29. T-tDNA_Cy5	Cy5-TTTATCAAAAAGAGTATTGACTTAAAG TCTAAACTATAGGATACTTACAG	50 nt fluorescently labeled target DNA for T-guide
30. T-guide_14Cy3	TTTAGACTTTAAG(dT-Cy3)CAAT	fluorescently labeled guide with 5'-T, forms pair with T-tDNA
31. 10nt guide	GTTAGACTTT	guide forms 5'-G pair with G-tDNA
32. 12nt guide	GTTAGACTTTAA	guide forms 5'-G pair with G-tDNA
33. 14nt guide	GTTAGACTTTAAGT	guide forms 5'-G pair with G-tDNA
34. 16nt guide	GTTAGACTTTAAGTCA	guide forms 5'-G pair with G-tDNA
35. 20nt guide	GTTAGACTTTAAGTCAATAC	guide forms 5'-G pair with G-tDNA
36. 22nt guide	GTTAGACTTTAAGTCAATACTC	guide forms 5'-G pair with G-tDNA
37. F1 guide	TTTTAAGTTGTTGATTTT	plasmid cleavage assay
38. R1 guide	TAAAAATCAACAACTTAA	plasmid cleavage assay
39. F2 guide	TAATAATTGACGATATGA	plasmid cleavage assay
40. R2 guide	GATCATATCGTCAATTAT	plasmid cleavage assay
41. F3 guide	GATTTTTAAGTCTTTCGC	plasmid cleavage assay
42. R3 guide	ATGCGAAAGACTTAAAAA	plasmid cleavage assay
43. F4 guide	TTCGATATCAAGCTTATC	plasmid cleavage assay
44. R4 guide	GGTATCGATAAGCTTGAT	plasmid cleavage assay
45. F5 guide	CTTTCGCATTTCGCCCTA	plasmid cleavage assay
46. R5 guide	TATAGGGCGAAATGCGAA	plasmid cleavage assay
47. F6 guide	AACGGTGAATCCGTTAGC	plasmid cleavage assay
48. R6 guide	TCGCTAACGGATTCACCG	plasmid cleavage assay
49. pSRKKm-t insert	GCCGCCAGATCTTCCGGATGGCTCG AGTTTTTCAGCAGATCCATCCCAATC GACTCAGGCCATCCCAATCGACTCAG GTGACTAGTCGAGCTGTCCCTCTCGA TGGCTGTAAGTATCCTATAGGTTAGA CTTTAAGTCAATACTCTTTTTGATAAA TTTTCGGGATCTGGATCCAAATAGAA TTCATCTTTCTAGAAGATCTCCTACAA TATTCTCAGCTGCCATGG	sequence of the insert in pSRKKm_t, the site complementary to the F7R7 gDNAs is underlined
50. F7 guide	GTTAGACTTTAAGTCAAT	plasmid cleavage assay
51. R7 guide	ATTGACTTAAAGTCTAAC	plasmid cleavage assay